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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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THE CHEMILUMINESCENCE DETECTION OF GLUCOSIDES IN WHEAT SAMPLES

by

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To all the Nieman group members, past and present, thank you for all the "bits of nugget" left behind, for those of us who follow, to learn from.

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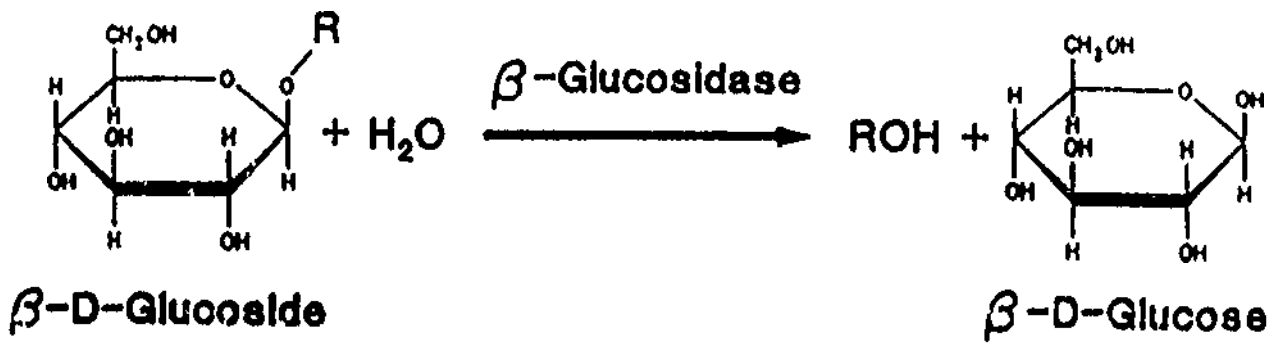
Introduction

The work done on this thesis is primarily concerned with the detection of glucosides using chemiluminescence (CL) detection. The interest in detecting glucosides lies in a plant's ability to metabolize certain herbicides. Many plants produce β-D-glucosides by metabolizing foreign molecules via conjugation with β-D-glucose to form the corresponding glucoside. If a plant is unable to metabolize the foreign molecule in this or a similar manner, the substance will be toxic to the plant. Therefore, it is possible to determine what type of plant will be effected by a certain herbicide by detecting the types of glucosides present in the species. This thesis describes improvements to a method by which CL can be used in the determination of β-D-glucosides in wheat samples.

In order to detect glucosides by CL, the glucosides must first be converted into H_2O_2. This process is accomplished by two immobilized enzyme reactors (IMERs). The glucoside is first flowed through a β-D-glucosidase IMER in which glucose is generated from the glucoside. The glucose is then passed through a glucose oxidase IMER in which H_2O_2 is generated from the glucose. The H_2O_2 then reacts with the chemiluminescent luminol to produce CL emission. The intensity of the light emission is a function of the H_2O_2 concentration and in turn the glucoside concentration. See Figure 1.

The chemiluminescence reagent used in this work is luminol (5-amino-2,3-dihydro-1,4-phthalazinedione). Luminol undergoes a chemiluminescence reaction when it is oxidized by a strong oxidant. For this work, H_2O_2 is the oxidant. For luminol to be oxidized by H_2O_2, a catalyst is required. Horseradish peroxidase and hemin are catalysts commonly used in the Nieman research group.



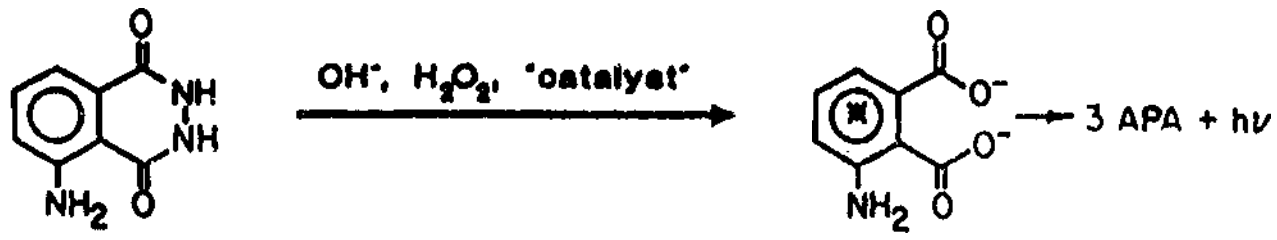


Figure 1. Reaction Scheme for the Determination of β-D-Glucosides Via Luminol Chemiluminescence. Reprinted from P.J. Koerner Jr., "Investigation of the Luminol Chemiluminescence Reaction as a Post-Column reaction Detector for HPLC," PhD Thesis, University of Illinois, Urbana, IL (1988)

There are several advantages to using chemiluminescence as a detection scheme. The instrumentation needed to perform the analysis is relatively simple. The reagents used are inexpensive and quite safe to handle. The major advantage is the linearity and sensitivity of the response to analyte concentration. A chemiluminescence working curve for hydrogen peroxide shows linearity from concentrations of 10^{-3} to 10^{-6} M and a detection limit of 10^{-7} M concentration. See Figure 2.

The latter part of the PhD research of Philip Koerner dealt with developing the HPLC-IMER-chemiluminescence detection scheme for glucosides. This approach yielded glucoside detection limits of about 0.1 μM for p-nitrophenyl-β-D-glucoside. When this technique was applied to the determination of glucosides in wheat extract, some unidentified component of the wheat extract matrix caused a severe interference and resulted in near complete suppression of the glucoside signal at concentrations well above the detection limit. It was the goal of my research to identify and eliminate this particular interference.[1]

Preparation of the Wheat Sample

Before attempting any analysis for glucosides in the wheat sample, the glucosides must first be separated from the wheat matrix. This process involves three steps: homogenization, filtration, and extraction. The preparation was performed on a sample of wheat foliage which was grown under controlled conditions by DuPont. The sample was untreated by herbicides or pesticides. The wheat had already been processed by chopping it in a blender with dry ice. The chopped wheat was stored in the freezer until use. The sample preparation and cleanup steps were adapted by Philip Koerner from procedures developed by the Agricultural Products Department at DuPont.

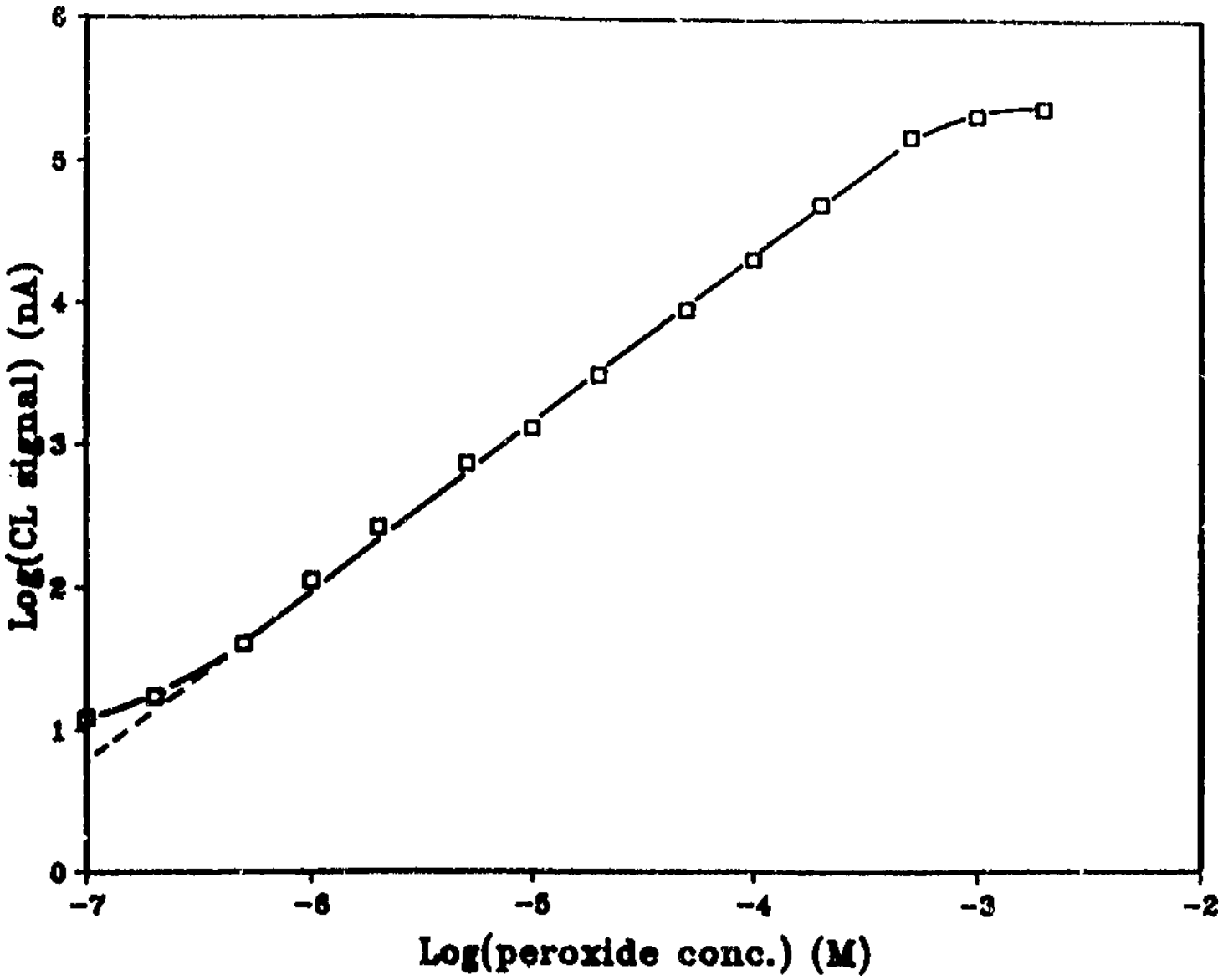


Figure 2. Hydrogen Peroxide Working Curve Using Luminol Chemiluminescence Detection Scheme. Reprinted from P.J. Koerner Jr., "Investigation of the Luminol Chemiluminescence Reaction as a Post-Column Detector for HPLC," PhD Thesis, University of Illinois, Urbana, IL (1988)

The first step was to weigh out approximately 5.0 grams of the wheat foliage into a 250 mL wide-mouthed plastic bottle. The wheat was taken up in 175 mL of methanol and homogenized for two minutes using a 4.0 mm probe fitted on a Kinematica homogenizer (model PCU 1). The solution was then placed in a Sorvall Superspeed RC-2B centrifuge for twenty minutes at a speed of 4000 rpm. The solution was then decanted through a 75 mL Bond Elut reservoir fitted with two 20 µm polyethlene frits. A dark green solution was collected in a 500 mL round-bottom flask. The solution was evaporated until 2 mL of residue remained. The solid mass collected after decanting was taken up in 100 mL of methanol and ultrasonicated for 30 minutes. This solution was centrifuged again for twenty minutes at 4000 rpm. The solution was decanted through the 20 µm frits into the corresponding round-bottomed flask containing the residue. The solution was evaporated to dryness and stored in the refrigerator until the next step could be performed.

The next step in the cleanup was to remove interferants which were ionic in form at pH 4.7. The procedure involves the use of Bond Elut PRS (propylsulfonic acid) columns (500 mg, 2.8 mL column volume, Analytichem). Before the filtration could be performed, the PRS columns had to be activated. Each column was first treated with two column volumes of hexane and dried under an N_2 flow for one minute. The column was then treated with two column volumes of methanol and dried under air suction. The third step was to treat the column with two column volumes of water and air dry. The columns were then put aside until just prior to use. When needed, the columns were conditioned with two column volumes of 10 mM sodium citrate (pH 4.7). The level of the sodium citrate was kept 5 mm above the bonded phase to avoid drying of the column. Three columns were used for each 100 mL sample.

The filtration apparatus was assembled using a 75 mL reservoir fitted with two 20 µm polyethylene frits attached to the top of a Bond Elut PRS column using the supplied adapters. The

PRS column was attached to a filtration flask (250 mL) attached to the laboratory vacuum supply. A valve was installed between the reservoir and PRS column to allow the PRS column to be changed without losing any of the sample.

The sample was taken up in 100 mL of the sodium citrate buffer. One third of the sample was then added to the reservoir. The valve was opened and the vacuum turned on. The sample was allowed to drip at a rate of one drop per second. This rate allowed the preceding drop to be absorbed onto the bonded phase. A dark green band was formed in the top 3 mm of the PRS column. The solution changed in color from a dark green to a light greenish-brown. After each portion of the sample was filtered through the column, the column was changed. This allowed the filtration to proceed at a reasonable rate and also insured the quality of the filtration.

After filtration was complete, the solution was qualitatively transferred to a clean 500 mL round-bottom flask. The solution was stoppered and stored in the refrigerator until the extraction could be performed.

The next step involved the removal of most of the organic material from the sample. The pH of the sample was adjusted to pH 2.5 with HCl. The aqueous solution was extracted with two 90 mL, two 50 mL, and two 25 mL aliquots of chloroform and the light green chloroform extract discarded. The aqueous phase was then treated with three 100 mL aliquots of n-butanol and the light green aqueous phase discarded. The n-butanol was transferred to a 500 mL round-bottom flask and the solvent removed on a rotary evaporator at 60 ° C.

The samples were reduced to a viscous, yellow-brown residue. The residue was dissolved in methanol and quantitatively transferred to a 100 mL pear-shaped flask. The solvent was removed

and the residue taken up in ten 1 mL aliquots of methanol delivered by an Eppendorf syringe. Each flask was stoppered and stored in the refrigerator.

Matrix Problem

In Philip Koerner's research, it was discovered that although a glucoside such as p-nitrophenyl-β-D-glucoside will elute at a time when no wheat matrix component is producing a signal, it is impossible to detect that glucoside even at concentrations well above the detection limit in the absence of the wheat matrix.[2] To investigate this phenomenon further, an experiment using flow injection analysis was performed with multiple glucoside injections, a single wheat sample injection, followed by repeated injections of the glucoside. The data confirms that something present in the wheat matrix is acting as an interferant in the CL detection scheme. See Figure 3. The interference is reversable and only temporarily effects the detection of the glucoside. A number of scenarios were proposed by Koerner as to the cause of the problem. One possible problem may be a component in the wheat matrix is absorbing the CL emissions. A second theory suggests that an interferant is present that interfers with one or both of the enzyme columns. The third possibility is an interference of the CL reaction.[3]

Absorbance Spectra

To investigate the possibility of a species absorbing the CL emission, an absorbance spectrum for the wheat sample was taken in the 300 to 600 nm region. At particular interest is the 425 nm wavelength region because this is the emission maximum for the luminol reaction. The instrument used in this procedure was a Hewlett-Packard Model 8450a Diode Array Spectrophotometer. The photo cells were made of quartz with a 4 mm width and a 1 cm path length. A 100 µL aliquot of

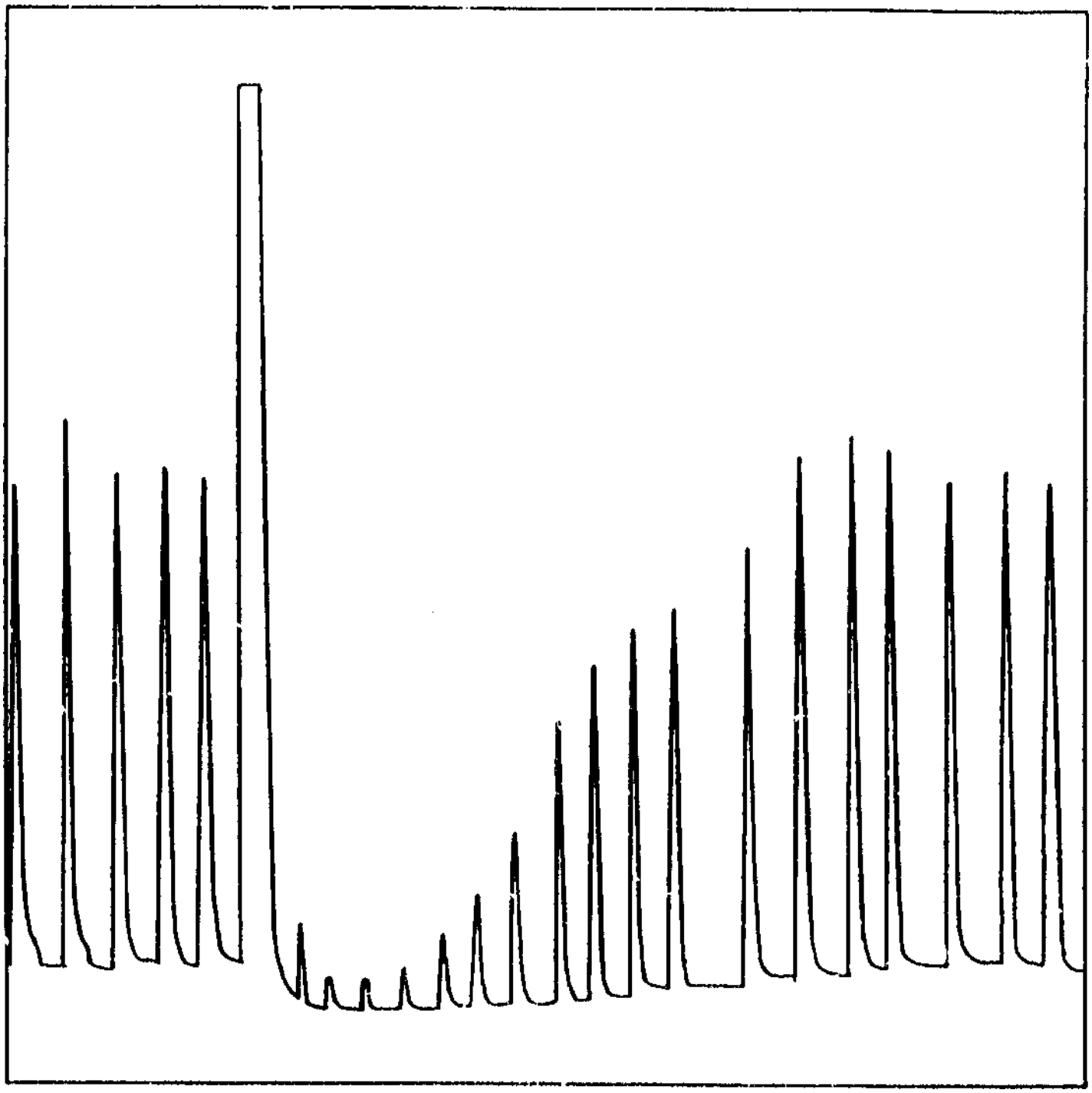


Figure 3. Data Tracing Showing Temporary Effects of the Wheat Matrix on the Detection of Glucosides

the sample was placed in a 10 mL volumetric flask and the methanol removed under an N_2 stream. The residue was taken up in the mobile phase (25% Acetonitrile/75% 1 mM KH_2PO_4 (pH 6.5)). The photo cells were washed with Millipore water and mobile phase and filled with sample. A spectrum was taken for the desired range. The procedure was repeated using a sample prepared from a 500 µL aliquot of the wheat matrix.

The sample was found to have an absorbance maximum at 339 nm, but showed little absorbance at 425 nm. Therefore it was determined that the sample was not absorbing the CL emission to a noticeable extent. See Figure 4.

Flow Injection Analysis Using Chemiluminescence Detection

Another possibility responsible for the detection problem is a large molecule, such as a protein, may be reducing the efficiency of the IMERs by binding or blocking access to the active sites. To determine if this is the case a series of experiments using flow injection was performed. The system consisted of a Rainin peristaltic pump with 4 channel capability set to deliver 2.0 mL/min. One channel was used for the 0.1 mM phosphate buffer (pH 6.5) and another channel delivered the luminol reagent solution. The analyte of interest was injected into the buffer stream by a Rheodyne Model 5020 80 µL sample injection loop. The analyte then passes through the two IMERs which convert the analyte to hydrogen peroxide. The two streams are joined at a mixing tee and flowed through a 5.5 cm strip of air permeable tubing to remove any air bubbles in the flow stream. The stream then proceeds into the flow cell made of a transparent plexiglass face and a white teflon reflective backing. The flow cell used teflon spacers to give it a 50 µL volume. An RCA Model 1P28 photomultiplier tube biased at -958 V detected the light emissions. The PMT was amplified by a Pacific Model 126 photometer and recorded on a Curken stripchart recorder. See Figure 5.

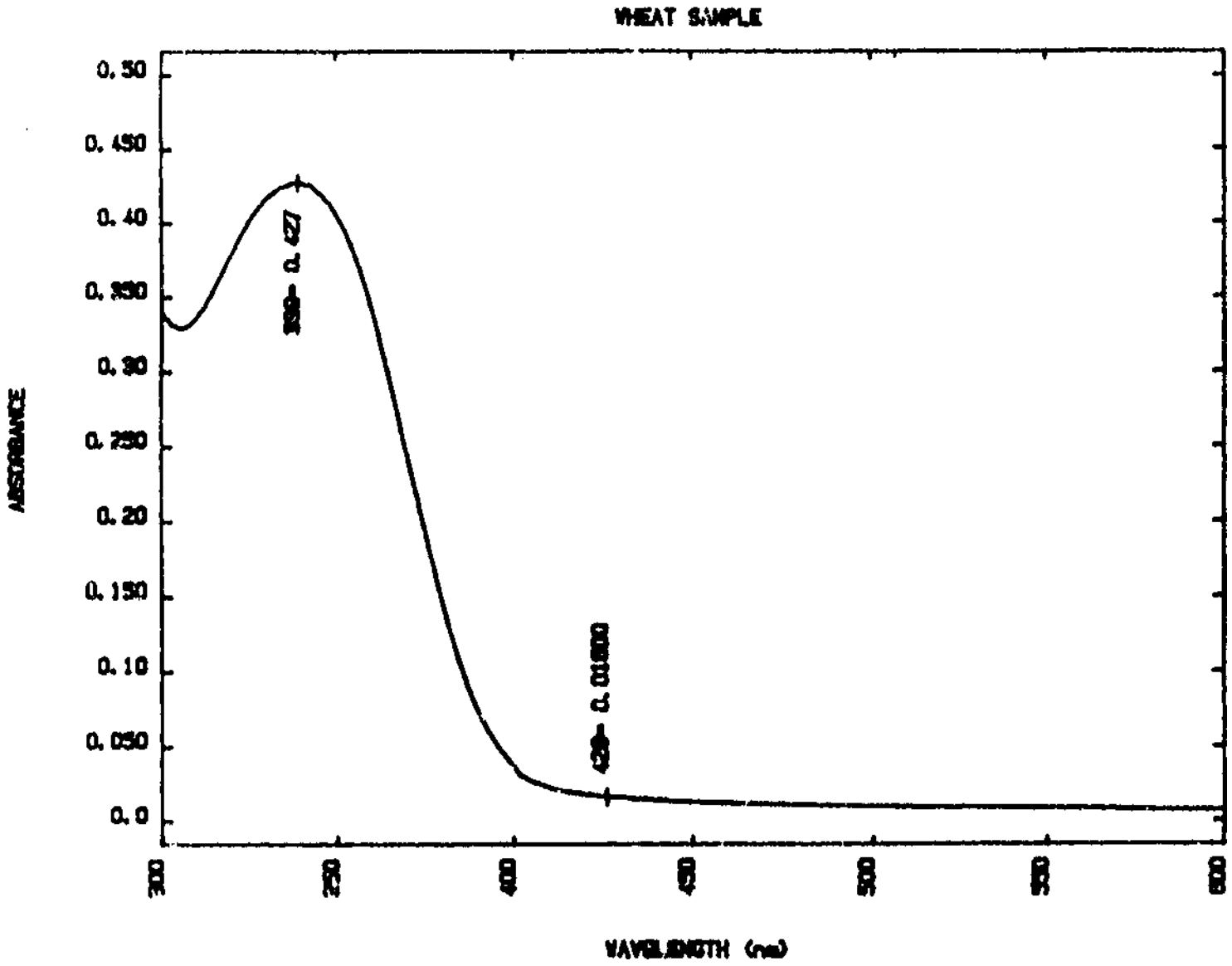


Figure 4. **Absorbance Spectrum of the Wheat Sample Obtained for the 300 to 600 nm Wavelength Region**

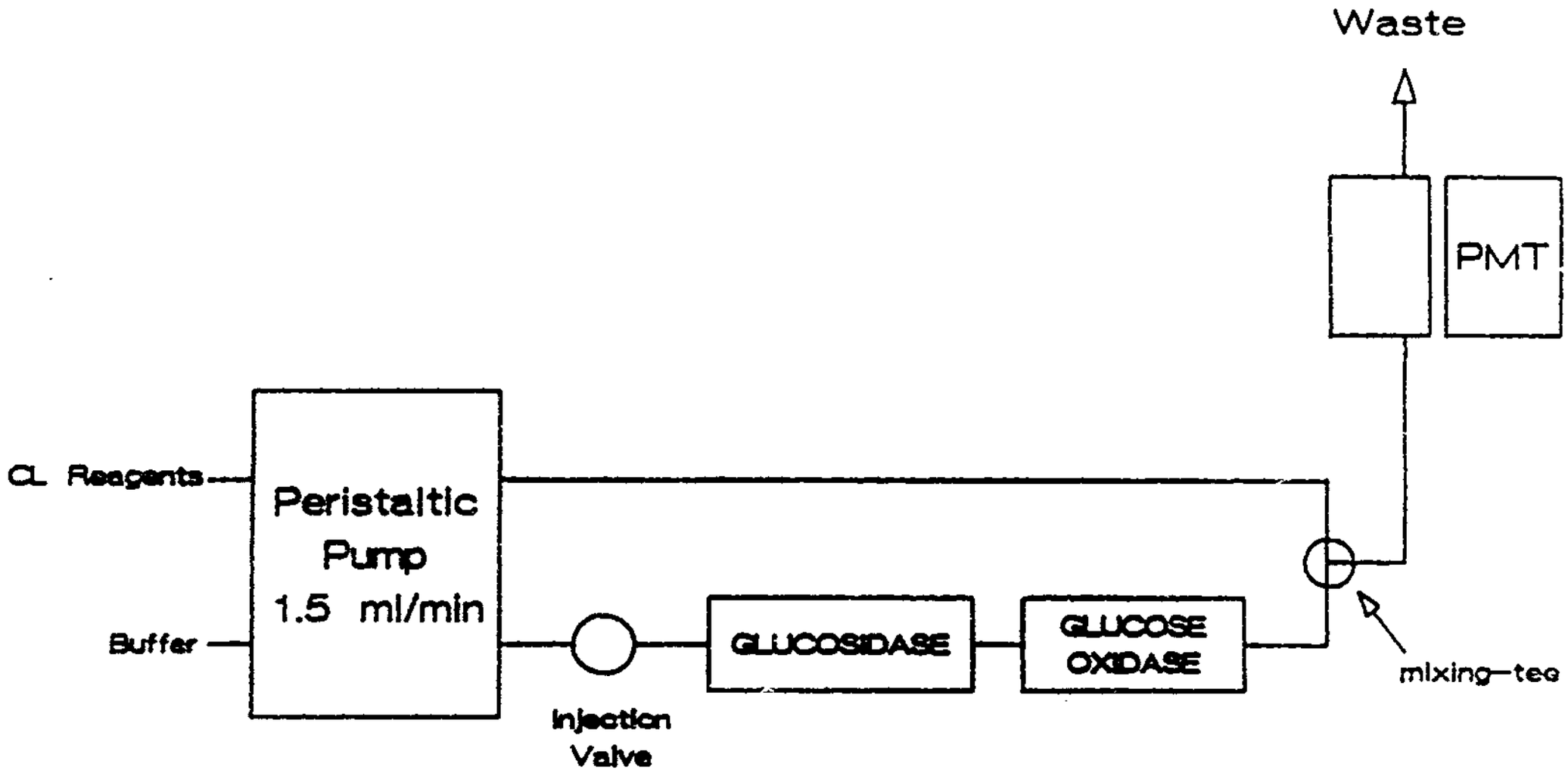


Figure 5. Diagram of the Flow Injection System Used for the Determination of β-D-glucosides

The luminol reagent solution consisted of 0.4 mM luminol (Aldrich), 8 mg/mL horseradish peroxidase (Sigma Chemical Co.), 2 μM hemin (Sigma Chemical Co.), and 0.1 M monobasic/ dibasic potassium phosphate (Mallinckrodt) buffered at pH 11.6. The phosphate buffer solution contains 1 mM potassium phosphate buffered at pH 6.5.

In the first experiment, a 10 μM solution of p-nitrophenyl-β-D-glucoside (Sigma Chemical Co.) was prepared just prior to conducting the experiment. A 1 mL aliquot of the wheat sample was placed in a 2 mL volumetric flask and the methanol removed. The residue was taken up in the phosphate buffer solution. The photomultiplier tube, recorder, and photometer were allowed to warm up at least five minutes before any injections were made. A series of glucoside injections were made to establish a baseline and peak intensities. An injection of the wheat sample was made and successive injections of the glucoside proceeded.

In the next experiment, a 10 μM solution of glucose (Sigma Chemical Co.) was prepared the day before the experiment was done to allow the solution to reach equilibrium between the D and L conformations. The β-D-glucosidase IMER was removed from the flow stream since only the glucose oxidase is needed to convert glucose to hydrogen peroxide. By removing the b-D-glucosidase IMER, we can examine if it is responsible for the detection problem. The glucose was injected in series to establish a baseline and peak intesities. An injection of the wheat was made followed by more glucose injections.

To examine if the problem lies in the glucose oxidase IMER, the IMER was removed from the flow stream and a 10 μM solution of hydrogen peroxide (E. K. Industries) used as the analyte. The same injection procedures were followed as in the two previous experiments.

In the first experiment, the data show that prior to the wheat injections the baseline remained very stable and the peak intensities were reproducable. After the wheat injection, the baseline fell dramatically and the peak intensities diminished to only 5% of their original height. The baseline and peak heights eventually returned to normal after 30 to 40 minutes of glucoside injections. See Figure 6. In the experiment where the β-D-glucosidase column was removed, the data show the same reproducibility and stable baseline before the wheat injection, but this time the baseline does not fall and the peak intensities return to normal after only 5 to 10 minutes. The peak intensities reduced to 15% of their original height, but only for the first injection. See Figure 6. The experiment in which both IMERs were removed showed very similar results. See Figure 6. From this data, it is thought that at least part of the detection problem involves the β-D-glucosidase IMER.

Flow Injection Analysis Using Amperometric Detection

To confirm the conclusion that part of the problem lies in the β-D-glucosidase IMER, the original experiment was repeated using amperometric instead of chemiluminescence detection. The flow system was set up using only the buffer stream connected to amperometric detection cell downstream from the IMERs. See Figure 7. The detection cell was constructed using a platinum working electrode (+500 mV vs Ag/AgCl), a stainless steel counter electrode, an Ag/AgCl reference electrode (downstream), and a (green) 4.0 μL volume spacer. See Figure 8. The cell was attached to an IBM EC/230 amperometric detector. A 10μM p-nitrophenyl-β-D-glucoside solution was prepared just prior to the experiment. The inlet line attached to the cell first to bleed air bubbles out. The outlet line was attached to the downstream reference electrode and again the bubbles were bled out. The cell was then turned on and allowed to stablize for 20 minutes. The same procedure for the injection sequence was used as in the chemiluminescence experiment.

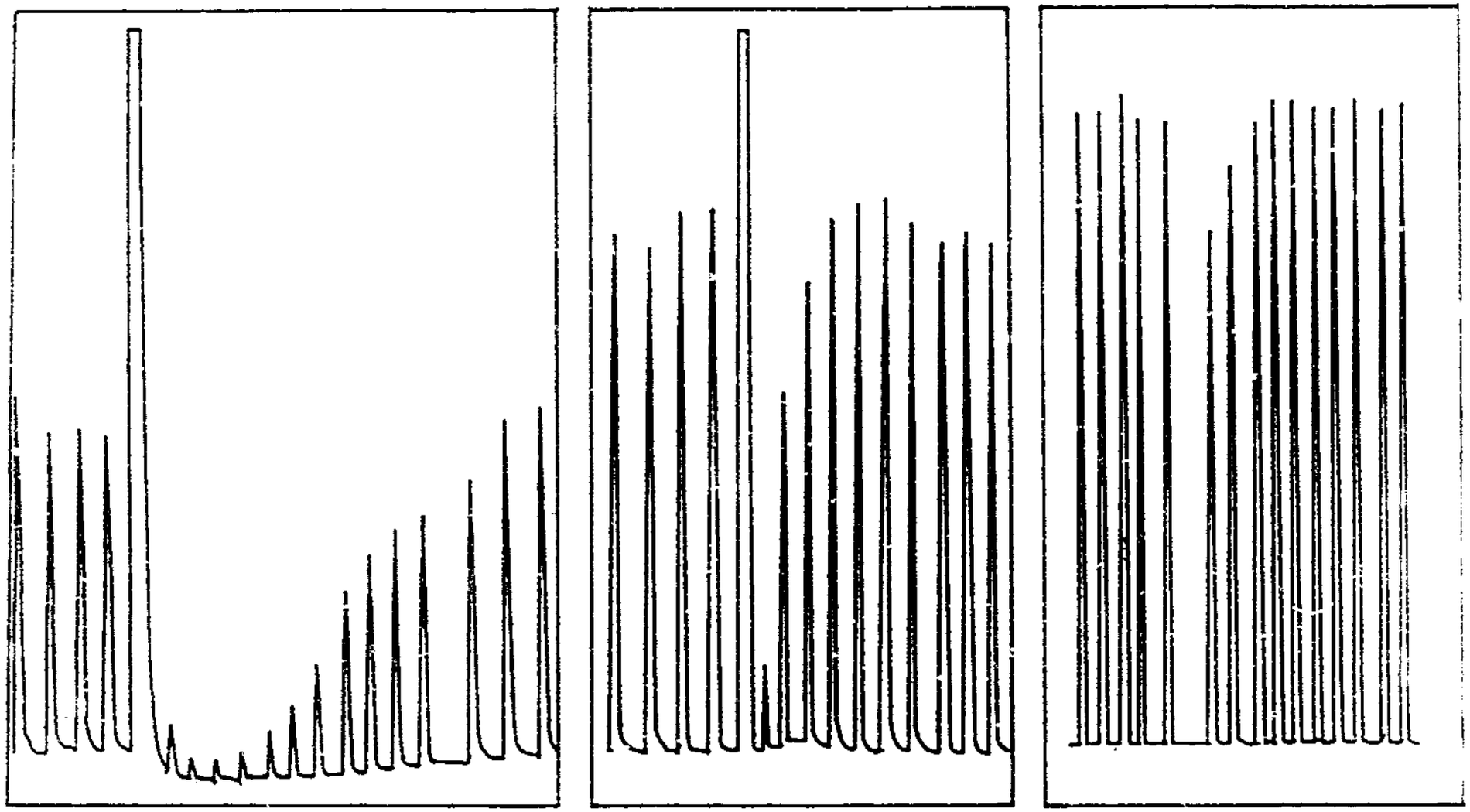


Figure 6. Data Tracing Showing Effects of the Wheat Matrix on the Chemiluminescence Detection Scheme. A) Glucoside Injections Using Both IMERs. B) Glucoside Injections Using the Glucose Oxidase IMER. C) Hydrogen Peroxide Injections Without any IMERs.

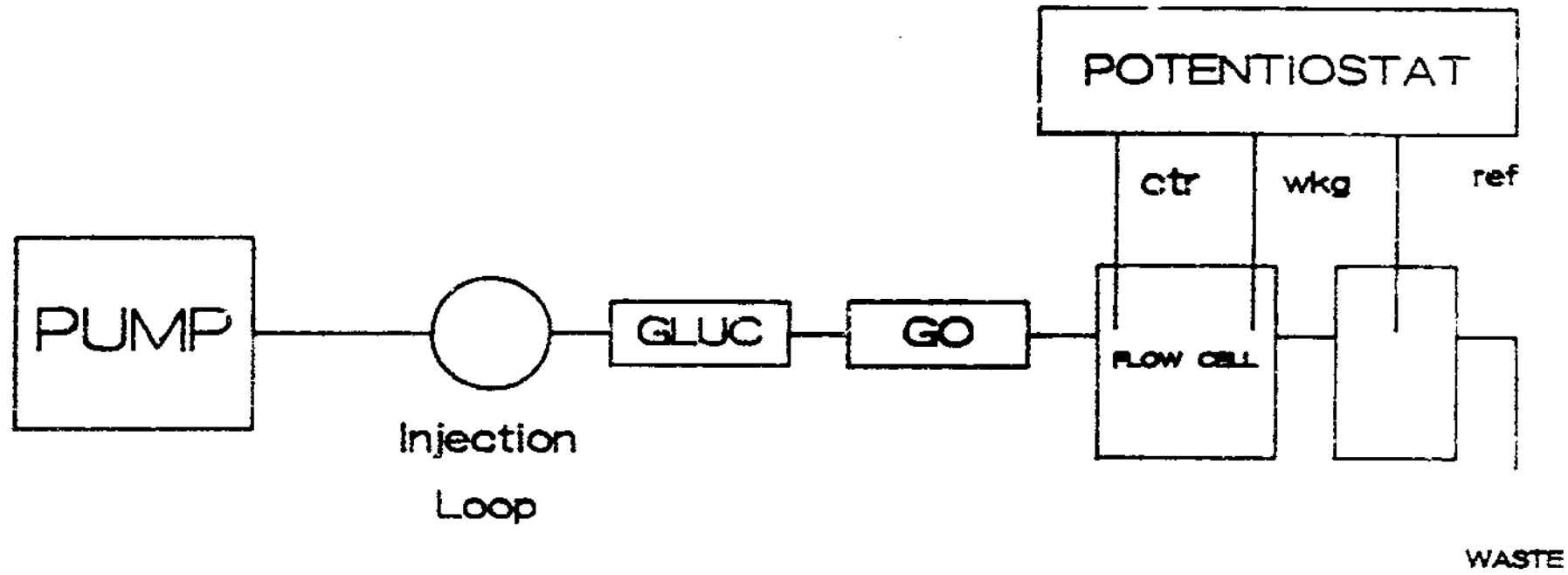


Figure 7. Diagram of the Flow Injection System using Amperometric Detection

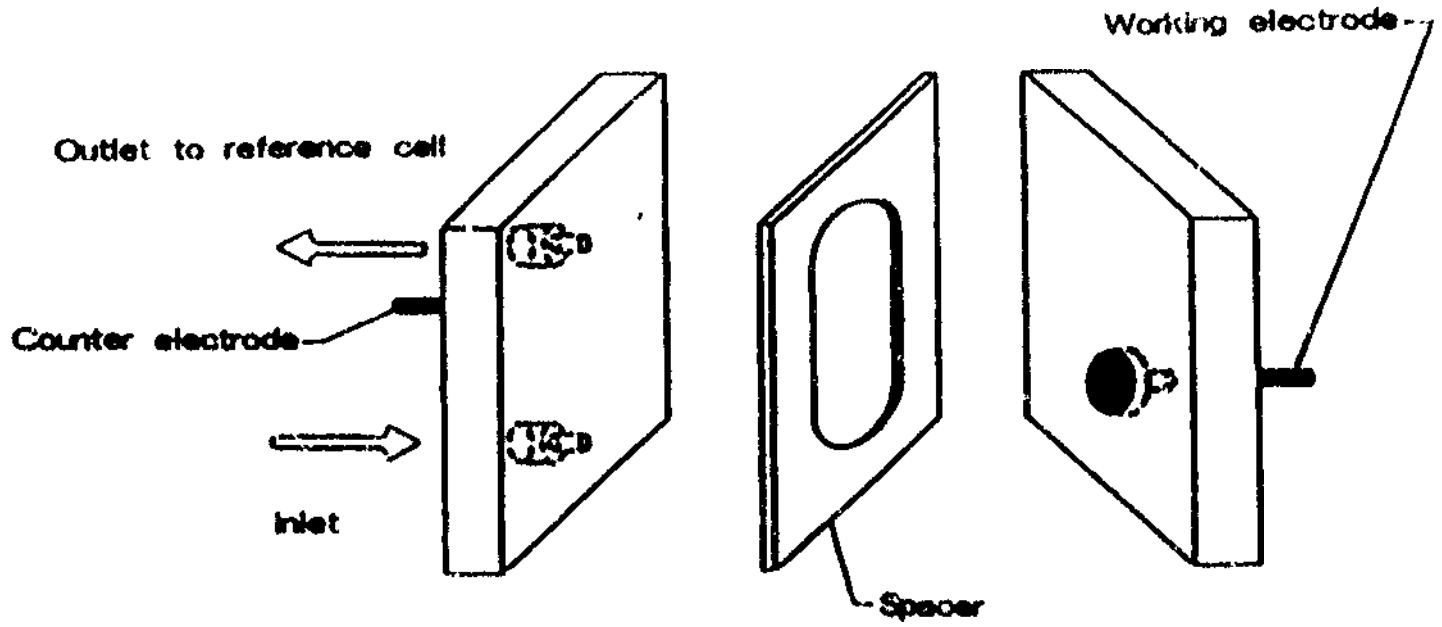


Figure 8. Flow Cell Used for the Amperometric Detector

The data obtained in this experiment was quite different than that obtained for the CL detection. The peak intensities and baseline prior to the wheat injection were again stable and reproducible. But after the wheat injection, the baseline rose slightly and the peak intensities were only diminished to 80% and never returned to their original intensity. See Figure 9. From this data, it would appear that there is little problem in the IMER column and interference with the chemiluminescence plays a major role in the detection problem. Because two different experiments give two different results, it was felt that the best possible solution would be to attempt the separation of the interferences from the glucosides.

HPLC Detection of Glucosides in Spiked Wheat Samples

The problem associated with the detection of glucosides in a wheat sample using the enzyme reactor / CL approach remains one of temporarily reduced sensitivity after the wheat matrix transverses the detector. By using a switching valve located at the end of an LC column, the interferants perhaps could be diverted to waste and the glucosides allowed to pass through the IMER columns.

An experiment was conducted on the flow injection system to find out if any problems might arise from stopping the flow to the IMERs. A switching valve was placed downstream from the injection loop, but before the IMERs. Glucoside sample injections were made and the time delay was varied to produce proportional peak heights. The data produced was reproducable and no problems with the peak intensities or baseline were discovered. The HPLC system was assembled using an Altex Model 110A pump, a silica precolumn, an Altex Model 210 injection loop (20 µL), a Partisil ODS-2 guard column (70 x 2.0 mm i.d.), a high pressure in-line filter, and a DuPont Zorbax ODS analytical column (5 µm, 250 x 4.6 mm i.d.). The outlet of the analytical column was

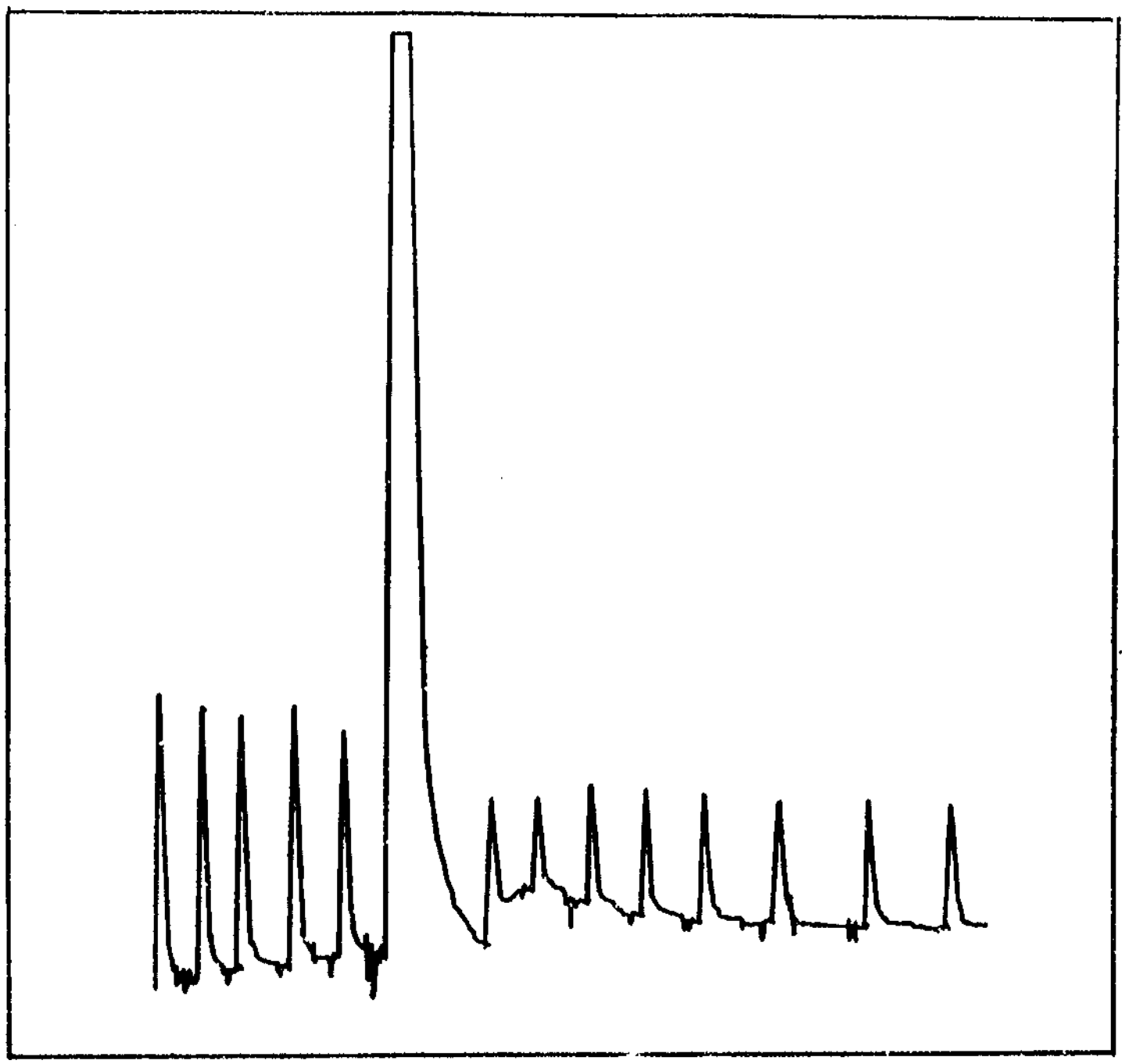


Figure 9. **Data Tracing Showing the Effects of the Wheat Matrix on the Amperometric Detection of Glucosides**

attached to a 6-way Valco valve in which the outlet possibilities were to waste or to the IMERs. The valve was equipped with a manual and remote switching, only the manual switch was used in this work. The IMERs were joined to the luminol reagent line, delivered by a Rainin peristaltic pump at a rate of 1.0 mL/min, by a mixing tee. The steam entered the flow cell with a 50 µL volume. The flow cell was placed adjacent to a Hamamatsu 1P28 photomultiplier tube where the CL was detected. The signal was amplified by a Pacific Precision Instruments model 126 photometer and recorded on a Curken stripchart recorder. See Figure 10. The mobile phase used contained 25% acetonitrile (v/v) in 1 mM phosphate buffer at pH 6.5 and was delivered at a rate of 1.0 mL/min.

The first step was to measure the retention time of p-nitro-β-D-glucoside on the Zorbax column. A 100 µM solution was prepared just prior to the experiment. The instrumentation was turned on and allowed to warm up for 10 minutes while the flow was being put through the IMERs. The valve was switched to waste and the glucoside was injected. A time delay was measured and the valve was switched to the enzyme columns. The time delay was increased until the peak intensities began to decrease. The retention time on the Zorbax column was calculated to be 215 seconds for p-nitro-β-D-glucoside. See Figure 11.

The next experiment examined the detection of this glucoside in a spiked wheat sample. A 1 mL aliquot of the wheat sample was placed in a 2 mL volumetric flask and the methanol was blown off under an N_2 stream. A 1 mL aliquot of 100 µM p-nitrophenyl-β-D-glucoside was added to the residue and the solution taken to volume with mobile phase. Therefore, the sample contained a 50 µM spike of the glucoside. A series of injections of the 100 µM glucoside were made to establish peak height. The valve was then switch to waste and the wheat was injected. The switch time delay was measured and the valve was then switch to the IMERs. The chromatogram

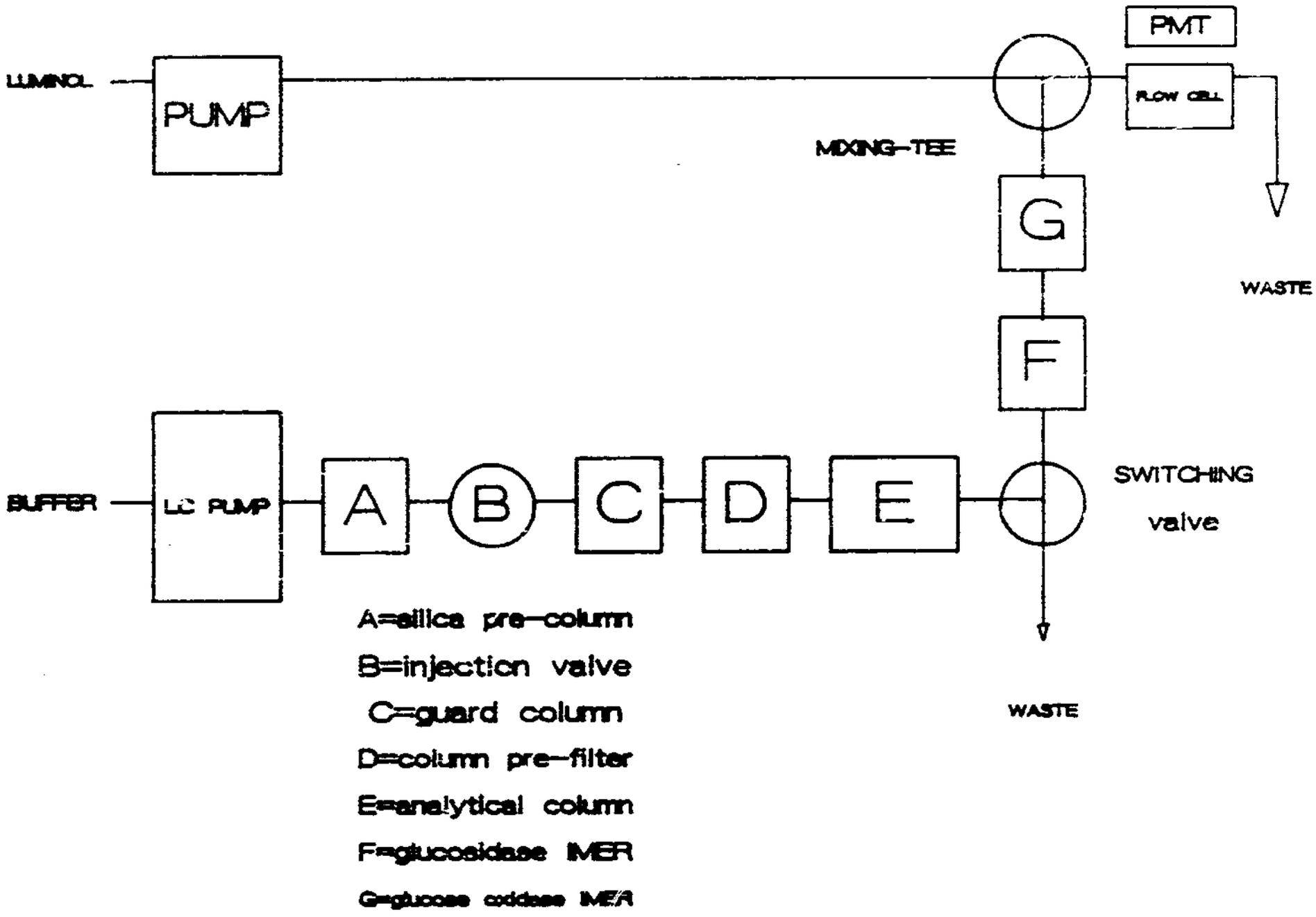


Figure 10. Diagram of the HPLC system Used for the Detection of Glucosides

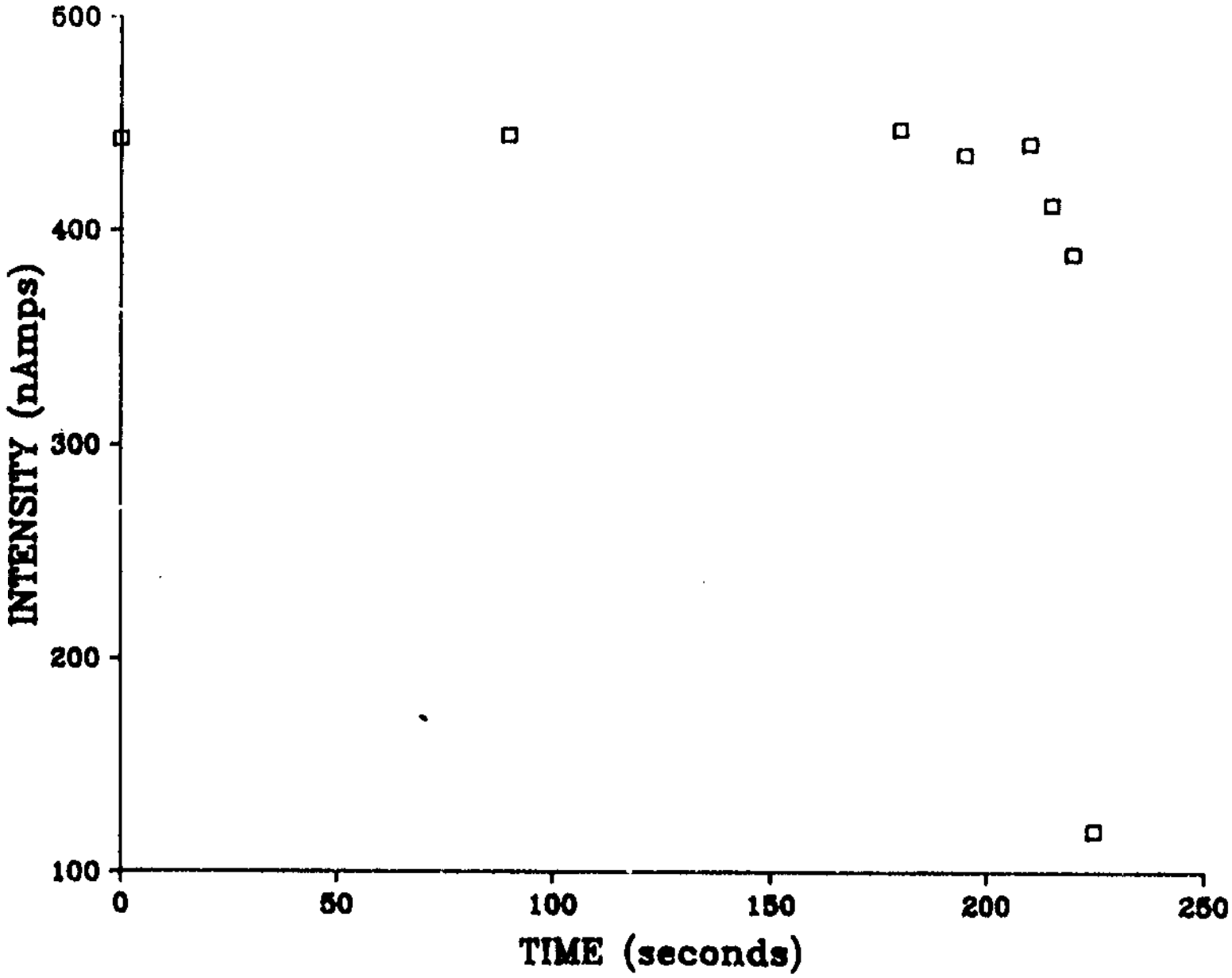


Figure 11. Graph Showing the Intensity as a Function of Switching Time

consisted of a single peak one-fifth the size of the 100 μM peaks. This result is somewhat disappointing. But if this peak is indeed the glucoside, it will be the first time that a glucoside has been seen in a wheat matrix.

To prove that the peak was the glucoside spike, an experiment was conducted involving several different concentrations of the glucoside spike in the wheat sample. Three wheat samples were prepared with spike concentrations of 10 μM, 25 μM, and 50 μM. Each sample was injected three times and the data compared to the data obtained by the injection of glucosides in the absence of the wheat matrix.

The data show that the peak intensities given by the spiked samples closely correlates with those given by glucoside standards. See Figure 12. This proves that this method of detecting glucosides in wheat samples will work for p-nitrophenyl-β-D-glucoside. It is thought that further investigation will show that the method is applicable to other glucosides as well.

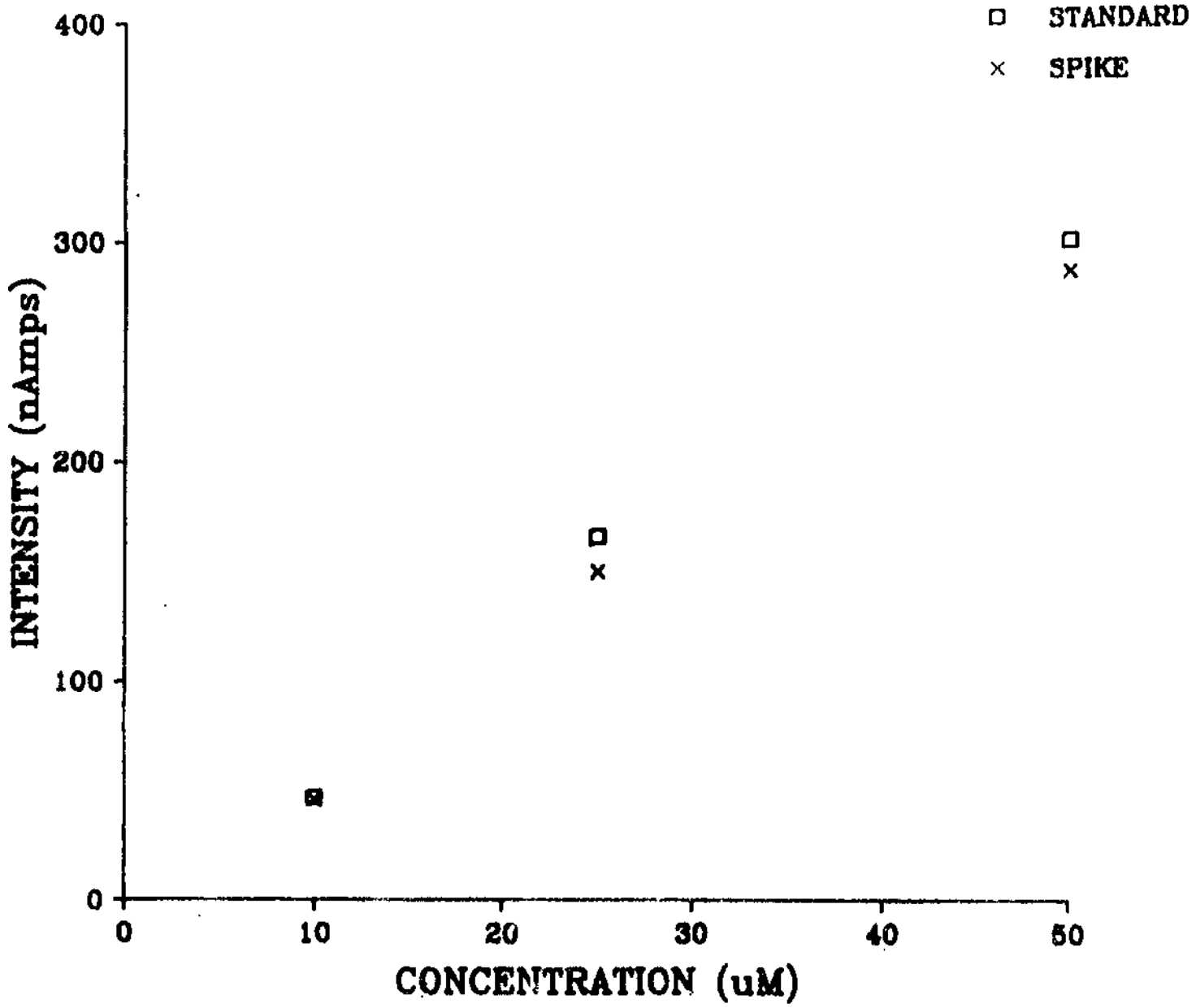


Figure 12. Graph Showing the Correlation Between Spike Intensity and Standard Intensity

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1. P.J. Koerner Jr., "Investigation of the Luminol Chemiluminescence Reaction as a Post-Column Reaction Detector for HPLC," PhD Thesis, University of Illinois, Urbana, IL (1988)

2. Ibid.

3. Ibid.